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Aspergillus niger as a Cause of Conjunctival Lithiasis in Children

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Abstract: Conjunctival lithiasis in children is very rare, with a single case reported in literature to the best of our knowledge. The present study constituted twelve cases of children presenting to Sarojini Devi Eye Hospital, Hyderabad between December 2013 to November 2017 with the complaint of formation of stones in their eyes. The cytological examination of material formed in the lower conjunctival fornix and that of the crushed stone was done. Microbiological culture and biochemical analysis of the stones was done. Conjunctival biopsies taken from the lower conjunctival fornix were fixed in 10% formalin, processed routinely, stained with Hematoxylin & Eosin and special stains. Stones were sent for biochemical analysis. The cytology, histopathology, and microbiology and biochemical findings are correlated. The cytohistological findings revealed branched fungal hyphae, calcium oxalate crystals and dark brown to black pigment deposits. A diagnosis of *Aspergillusniger* was made. Microbiological culture showed fungal growth consistent with *Aspergillusniger* on microscopy. Biochemical analysis showed that the stones were composed of calcium compounds. *Aspergillus niger* releases a mycotoxin, oxalic acid which complexes with calcium ions in tear fluid to precipitate as calcium oxalate crystals. Crystallogenesis is essential to stone formation. The probable mechanism of conjunctival stone formation and possible factors responsible for the rapid formation of conjunctival stones are analysed.

Keywords: Conjunctival lithiasis, children, *Aspergillusniger*, oxalic acid, calcium oxalate crystals.

INTRODUCTION

Aspergillus species is the most ubiquitous fungi seen in soil, water & decaying vegetations.[1] Most infections are attributed to *Aspergillusfumigatus, flavus* and *terreus.*[2] They affect the lungs, naso-orbital area, skin and may be disseminated [1]. *A. niger* is an uncommon cause of invasive aspergillosis. Concretions occurring as minute hard yellow spots in the palpebral conjunctiva due to accumulation of epithelial cells and inspissated mucus in depressions called Henle glands is sometimes seen in elderly people who suffered from trachoma.However rapid and spontaneous formation of stones of varying sizes in the conjunctiva of children is quite rare and puzzling. This study is taken up to know the underlying cause of conjunctival stone formation in children that helps the clinician plan specific treatment.

MATERIALS & METHODS

The present study constituted twelve cases of children presenting to Sarojini Devi Eye Hospital, Hyderabad between December 2013 to October 2017 with the complaint of formation of stones in their eyes. All the children came from lower socio-economic strata. The number of conjunctival stones formed on an average ranged from one to ten per day. The stones formed intermittently for few days with no stone formation for few days. There was no relevant past or family history. Routine hematological and biochemical investigations were done on all the patients. Soft material that appeared in the lower conjunctival fornix prior to hardening was smeared on slides and fixed in 90% alcohol. The hard stones were cleaned in xylene, crushed and made into fine powder, resuspended with egg albumin and smeared on slides. Cytology smears were stained with hematoxylin & eosin and Papanicoulou stains. Microbiological culture of the material and stones was done. Few stones were sent for biochemical analysis. Conjunctival biopsies from the lower fornix were submitted to histopathology. Formalin fixed tissues were routinely processed. Sections of four microns were cut from the paraffin wax embedded tissue blocks and stained with Hematoxylin & Eosin. Special stains, Gomori's Methanamine Silver (GMS) and Periodic Acid Schiff (PAS) were also done.

RESULTS

The ages of the children ranged from seven years to sixteen years. There were eight female children and four male children. All of them came from lower socioeconomic strata. They complained of foreign body sensation and pain prior to the appearance of conjunctival stone in the lower fornix. The stones ranged in size from 1mm to 1cm in different sizes, shapes and color varying from yellowish to white mottled and black to jet black. [Fig.1]

Fig-1: Gross appearance of stones formed in lower conjunctival fornix meas 1mm to 1 cm

General examination revealed moderately built and nourished children with no major illness. Examination of the eye was unremarkable except for mild to moderate inflammation of the lower conjunctival fornix. Visual acuity and fundus examination were normal. Routine investigations such as CBP, hematocrit, ESR and urine examination were within normal limits, but for mildly elevated eosinophil count in four children. Cytology smears of the conjunctival material showed cellular debris consisting of acute inflammatory cells and squamous epithelial cells. A refractile tangled mass of slender branched hyphae along with few conidia and areas of dark brown to black pigment deposits were seen. Clumps of needle shaped crystals and occasional dumbell shaped crystals were seen. Smears from the crushed stone material also showed crystalline material, fungal hyphae and pigment deposits [Fig.2] Diagnosis of *Aspergillus niger*was made on cytology. Microbiological culture of the stone on Sabouraud's Dextrose Agar showed fungal growth [Fig. 3].

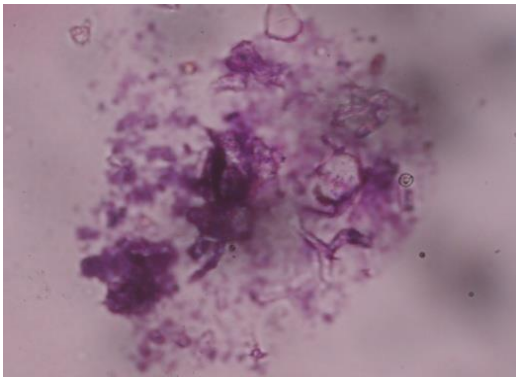

Fig-2: Cytology: Refractile hyphae, pigmentdeposits and crystalline material (H & E) 1000x' is the completesentence

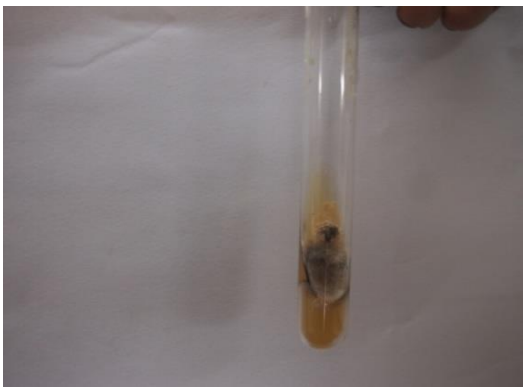

Fig-3: Fungal growth seen on culture

The fungus was identified as *A. niger* on microscopic examination with Lactophenol Cotton Blue (LPCB) stain [Fig.4]. No bacterial growth was seen. Biochemical analysis of the stones showed that they were composed of compounds of calcium. Tissue sections stained with hematoxylin and eosin revealed moderate to severe non-specific inflammation of the conjunctival submucosal stroma, refractile branched hyphae, pigment deposits and crystals. Special stains, GMS and PAS revealed fungal hyphae with acute angle branching and conidia within the conjunctival epithelial layers and superficial stroma [Fig. 5].

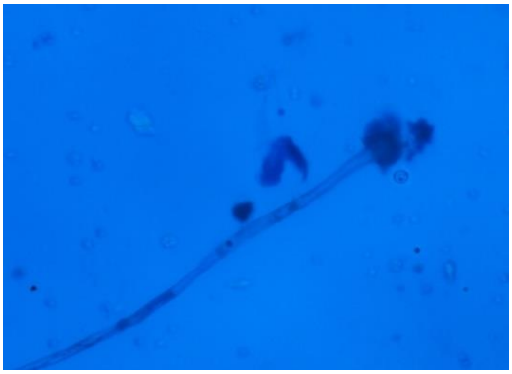

Fig-4: HPE Conjunctival biopsy showing branched fungal hyphae & conidia (GMS 1000x)

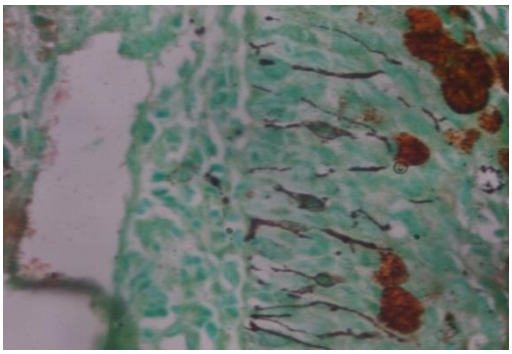

Fig-5: LPCB stain 400x shows *Aspergillus Niger*

Table-1: The Microbiology, Cytology, Histopathology & Biochemical findings are as shown

Diagnostic Test	Findings	Diagnosis
Microbiology Culture	Positive for fungal growth.	
Microscopy	Species identified on microscopy with LPCB stain (Lactophenol cotton blue)	*Aspergillus niger*
Cytopathology (H & E)	Tangled mass of refractile hyphae, Calcium oxalate crystals and dark brown pigment deposits	*Aspergillus niger*
Histopathology (H & E)	Sub mucosal inflammation, Refractile branched fungal hyphae and conidia, pigment deposits and crystals	*Aspergillusniger*
Histopathology (Special stains -GMS & PAS)	Fungal hyphae with acute angle branching and conidia within the epithelial layers & superficial stroma	*Aspergillus niger*
Biochemical analysis of stones	Calcium compounds	

DISCUSSION

Aspergillosis occurs in immune compromised individuals though; cases of invasive aspergillosis occurring in immunocompetent individuals have been reported in literature. A case of cutaneous aspergillosis caused by *A. niger* has been reported in an immune competent patient [1].Another case of extrapulmonary disseminated aspergillosis and a case of maxillary sinus mycetoma both due to *A. niger*occurring in immunocompetent individuals are reported in literature [3, 4]. *A. niger*is associated with otomycosis, cutaneous infections and pulmonary infections [5, 6].In the ocular tissues, aspergillus species have been implicated in a wide variety of infections characterized by either slow and asymptomatic infection or rapid uncontrollable progression [7].There are rare reports of *A. niger* infection complicating orbital exenteration in immunocompetent patients [8, 9].Patients in our study did not have any evidence of immunosuppression. *Aspergillusniger* prefers to grow in moist and warm environment and is commonly found in soil and plants [8,9].Hospital kitchens have been reported to be a source of this fungus [5]. In the present study, isolation of *A. niger* from the conjunctiva of children probably

suggests contamination due to poor personal hygiene and crowded environment.

A case of multiple white dacryoliths due to *Aspergillusfumigatus* is reported in literature [7]. There are isolated cases of conjunctival lithiasis in children reported in the news and media in India [10]. There are also isolated cases reported in the media from other countries such as Pakistan, Yemen and Nepal. However literature shows to the best of knowledge, only a single case reported by Lakshmi Narain in 1981[11]. Biochemical analysis in that case showed that the stones were composed of different compounds of calcium which is similar to the present study. Conjunctival biopsy in that case showed submucosal granulation tissue with perivascular calcification, whereas in the present study biopsy showed submucosal inflammation, branched fungal hyphae, black pigment deposits and calcium oxalate crystals. Deposition of calcium oxalate crystals in tissue is known to have strong association with aspergillus infections and more specific for *A. niger* strain [12].The presence of calcium oxalate and black pigment deposits in lung parenchyma is considered as evidence of *A. niger* infection [13].There was heavy calcium oxalate deposition on pathological examination in cases of *A. niger* causing invasive pulmonary aspergillosis [14, 15]. In the study of Choet al. the pathological specimen obtained by a transbronchial lung biopsy revealed numerous calcium oxalate crystals within the destructive lung lesions, but no fungal elements were identified [16].The association of calcium oxalate crystals and Aspergillosis was first described in 1973 by Nime and Hutchins [12, 17]. Oxalic acid generates local oxidants that cause cell injury and tissue destruction, including blood vessel destruction [16, 17]. Although calcium oxalate is not always detected in patients with Aspergillus infection, its presence is considered as characteristic of *A. niger* infection [13, 17, 18].The presence of calcium oxalate crystals should be considered as true sign of infection rather than colonization or contamination [12]. Identification of calcium oxalate crystals in sputum, fluids or tissue specimens should raise the possibility of aspergillosis caused by *A. niger*[19, 20].Also the presence of black pigment with calcium oxalate crystals could be a pivotal clue to diagnosis even in the absence of fungal hyphae or conidia [13, 21]. Wehmer first described oxalic acid as a fermentation product of *A. niger* in 1981[13, 16, 17]. Both *Aspergillusniger* and *fumigatus* produce oxalic acid which precipitates as calcium oxalate by reacting with calcium ions in tissue fluids or blood [12, 13, 16, 20]. Oxalic acid is a mycotoxin released by the fungus, formed as a side product of TCA cycle by enzymatic hydrolysis of oxaloacetate by oxaloacetate acetylhydrolase [13,16].

Having established *A. niger*as the underlying cause of stone formation, the exact mechanism of conjunctival stone by A.*niger* formation remains to be determined. Studies on the pathogenesis of renal stone formation are reviewed in order to understand the mechanism of stone formation. The pathogenisis of renal stone formation is a multistep process and includes nucleation, crystal growth, crystal aggregation and crystal retention [22].Nucleation is the formation of a solid crystal phase in a solution [23-25]. The process of nucleation in a pure solution is known as homogenous nucleation [23]. The process of nucleation that occurs over an existing surface such as epithelial cells, cell debris, RBCs etc. is known as heterogenous nucleation. Experimental studies demonstrated that injury from free radicals may result in sloughed membrane fragments providing a suitable nidus for nucleation. Stones result from a phase change in which dissolved salts condense into solids and this transformation is influenced by supersaturation. Crystal growth is the next step where several atoms or molecules in a supersaturated liquid start forming clusters. Crystal growth is determined by the molecular size and shape of crystals, physical properties of the material, supersaturation levels and pH. Epitaxy is a process where material of one crystal type is precipitated upon the surface of another whose lattice diemensions are almost identical [26]. Epitaxy is clinically important in stone formation. The next step is crystal aggregation in which crystals in solution stick together and form larger particle. Aggregation of particles in solution is determined by a balance of forces, some with disaggregating and some with aggregating effects. A small interparticle distance favors aggregation and the main force that inhibits aggregation is the repulsive electrostatic surface charge [22]. Crystal aggregation is more important than nucleation and growth because aggregation occurs within seconds [27]. Urolithiasis requires formation of crystals followed by their retention and accumulation in kidney by association of crystals to the renal epithelial cells [28]. Typically, supersaturation is not reached until the distal nephron, but in hyperoxaluric states, supersaturation for calcium oxalate is reached in the proximal tubule itself, which leads to crystal formation and contributes to stone formation [29].

Assuming similar mechanism for conjunctival stone formation, what causes instantaneous formation of conjunctival stones in comparison with the prolonged time taken for formation of renal stones is analysed. Urine contains several stone promoting factors and inhibiting factors, the imbalance between which has been suggested in the formation of renal stones. Calcium, sodium, oxalate, urate, cystine, low urinary pH and low urinary flow are some of the promoting factors for urinary stone formation. Cell debris, epithelial cell surfaces, protein aggregates and other crystals also act as promoters. Inhibitory factors include citrate, magnesium, pyrophosphate, Urinary prothrombin fragment 1, protease inhibitor, glycosaminoglycans, osteopontin, renal lithostathine and high urinary flow. These inhibitors cause growth inhibition of the stone at the level of nucleation,

aggregation or cell adherence. Although crystallogenesis is essential to stone formation, calcium oxalate stone growths in urine are sluggish [30]. To know the role of tear fluid in the formation of conjunctival stones the composition of tear fluid is studied. The major components of tear fluid are water, electrolytes, proteins (lysozyme, lactoferrin, lipocalin), secretory Ig A, albumin, IgG, lipids, Mucins, Defensins, collecctins *et al*. [31]. The electrolytes are principally Na^+, K^+, Cl^-, and HCO^- with lower levels of Mg^{2+} and Ca^2. The pH of the tear fluid in normal persons in the study of Norn MS was found to be 6.93+/- 0.24[32].

The following factors may possibly explain the rapidity with which stones form in the conjunctiva. Normally, tears constitute a thin film of fluid. Release of oxalic acid from *Aspergillusniger* leads to high oxalic acid concentration in the conjunctival sac which complexes with the calcium present in the small amount of tear fluid and achieves supersaturation levels quickly. This facilitates phase change and calcium oxalate crystal growth. Reduced tear secretion and changes in tear pH may contribute. The growth and aggregation of crystals into stones is potentiated by promoters of stone formation present in the tear fluid such as calcium, sodium, protein and surfaces of conjunctival epithelial cells. Presence of crystals and cell debris also act as promoters of stone formation. Compared to urine which has a large volume and numerous inhibitors of stone formation, tear fluid which is minute in volume seems to have very few inhibitors. Epitaxy seems to contribute to the formation of conjunctival stones. However further research to determine the molecular size and lattice diemensions of the crystals and factors determining crystal aaggregation need to be undertaken for clearer and more precise understanding of the mechanism of conjunctival stone formation.

CONCLUSIONS

The cytohistological findings in correlation with mirobiological culture in this study revealed the presence of *Aspergillusniger*, both in the conjunctival tissues and in the material formed in the conjunctiva. The biochemical analysis of the stones revealed calcium compounds. Oxalic acid is a mycotoxin released by *A. niger* which complexes with calcium in tear fluid to form calcium oxalate crystals. Crystallogenesis is essential for stone formation. Factors such as minute tear fluid volume, the pH of tear fluid and calcium levels facilitate supersaturation leading to high oxalic acid concentration in the conjunctival sac contributing to rapid formation of conjunctival stones. Further research is needed for more precise understanding of the mechanism of conjunctival stone formation. This study helps to specifically treat this mysterious disorder which has remained an enigma for physicians since decades, with topical and oral antifungal agents. Voriconazole is the drug of choice for treatment of *Aspergillusniger* infections.

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